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Extensive changes in innate immune gene expression in obese Göttingen minipigs do not lead to changes in concentrations of circulating cytokines and acute phase proteins

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Summary

The usefulness of Göttingen minipigs as models for obesity and obesity-related pathologies is well established. The low-grade inflammation associated with obesity involves a range of innate immune factors; however, to our knowledge, the impact of obesity on innate immune factor expression has not been studied in Göttingen minipigs. Therefore, we studied the expression of innate immune genes in liver and adipose tissues as well as serum concentrations of cytokines and acute phase proteins in obese vs. lean Göttingen minipigs. In the liver, of 35 investigated genes, the expression of nine was significantly different in obese pigs (three up-regulated, six down-regulated). Of 33 genes in adipose tissues, obesity was associated with changed expression of 12 genes in the visceral adipose tissue (VAT) (three up-regulated), 11 in the abdominal retroperitoneal adipose tissue (RPAT) (seven of these up-regulated) and eight in the subcutaneous adipose tissue (SAT) from the neck (five of which were up-regulated). Obesity-associated expression changes were observed for three genes in all adipose tissues, namely *chemokine (C-C motif) ligand 3-like 1* (up-regulated), *CD200 molecule* (down-regulated) and *interleukin 1 receptor antagonist* (up-regulated) with *interleukin 1 receptor antagonist* being the most highly regulated gene in both VAT and RPAT. Looking at patterns of expression across the three types of adipose tissues, obesity was associated with an increased number of acute phase proteins differentially expressed between adipose tissues and a decreased tissue-specific expression of cytokines and chemokines. In contrast to obese humans, no changes in serum concentrations of haptoglobin, C-reactive protein, serum amyloid A, tumor necrosis factor- α and interleukin 6 were found in obese Göttingen minipigs.

Keywords comparative immunology, immune responses, inflammation, mRNA, obesity, pig model, porcine models

Introduction

Obesity has become an important health problem worldwide and is associated with elevated risks of several life-threatening conditions, including cardiovascular diseases and type II diabetes (Nguyen & Lau 2012). Obesity is

associated with chronic low-grade activation of the innate immune system, resulting in a chronic inflammation of the adipose tissue, which is thought to lead to the development of these disease states (Bastard *et al.* 2006; Gil *et al.* 2007; Federico *et al.* 2010). A wide range of factors of the innate immune response have been investigated with regard to obesity [reviewed, for example in the study by Federico *et al.* (2010) and Gil *et al.* (2007)]. The pig has been proposed as an excellent biomedical model for obesity as it is very similar to man with regard to anatomy, physiology, biochemistry and metabolism (Litten-Brown *et al.* 2010), and the use of the Göttingen minipig as a human obesity (Johansen *et al.* 2001) and diabetes (Larsen *et al.* 2002, 2007) model has been demonstrated. To further elucidate the usefulness of

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the Göttingen minipig as a human obesity model, it is important to include other molecular changes associated with obesity. The objective of this study was therefore to elucidate the effects of diet-induced obesity on the gene expression of innate immune factors in the liver and in three types of adipose tissues in the Göttingen minipig and to relate this to human obesity.

Materials and methods

Animals, diets and sampling

The study was approved by the Danish Animal Experiments Inspectorate. Fourteen female Göttingen minipigs (seven lean and seven obese) originating from Ellegaard Göttingen Minipigs A/S were included in the study. With the exception of one pair of half siblings (same mother) split between the two groups and one pair of half siblings (same father) in the obese group, all pigs in the two groups were unrelated at the parent level. The pigs were ovariectomized at 9–15 months of age to avoid hormonal effects on feed intake, and they were housed at the University of Copenhagen (Taastrup, Denmark) from 1–2 years of age and forward. The obese pigs had previously been used in pharmacological studies with therapeutic peptides, but had been subjected to a suitable washout period, based on the knowledge of the half-life of the peptides, prior to this study. Lean pigs were fed minipig standard chow Altromin 9023 2×150 g a day and obese pigs had *ad libitum* access to minipig standard chow Altromin 9033 from the point of ovariectomization. The minipigs were euthanized with pentobarbital and exsanguinated at 41–47 months of age. Tissues were collected from the liver, subcutaneous adipose tissue (SAT) from the neck, mesenteric adipose tissue (VAT) and abdominal cranioventral retroperitoneal adipose tissue (RPAT) and snap-frozen in liquid nitrogen, and blood was collected for serum preparation. Tissue and serum samples were kept at -80°C until analysis.

Extraction of RNA

All samples were homogenized on a gentle MACS Dissociator (Miltenyi Biotec) in gentleMACS M tubes (Miltenyi Biotec). For liver samples, RNA was extracted using the RNeasy Lipid Tissue Midi kit (Qiagen) according to manufacturer's protocol. All samples were subjected to on-column RNase-free DNase digestion (Qiagen).

For the adipose tissues, an optimized procedure was developed as follows: RNA was extracted with phenol and chloroform: Approximately $\frac{1}{2}$ gram of adipose tissue was homogenized with 4 ml of QIAzol Lysis Reagent (Qiagen) on the gentleMACS Dissociator and then placed at room temperature for 5 min. The homogenate was centrifuged at 12 000 *g* for 10 min at 4°C , after which the fatty upper layer was removed. Chloroform (Merck) was mixed with the

remaining supernatant and placed at room temperature for 3 min after vigorous shaking for 15 s. One ml of isopropanol (2-propanol; Merck) was added to the upper, aqueous phase and was centrifuged at 12 000 *g* for 10 min at 4°C after 5 min at room temperature. The pellet was washed 2 times in cold 75% ethanol, with a centrifugation step of 8 minutes at 8000 *g* at 4°C between each wash and then dried at 55°C for 2 min. The RNA was dissolved in RNase-free water at 55°C for 10 min.

For all samples, the RNA yield was measured on the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.), and RNA integrity was measured with the Agilent RNA 6000 Nano Kit (Agilent Technologies) on the Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's protocol. Liver samples yielded RNA with an RNA integrity number (RIN) of 9.0 ± 0.1 , RPAT samples had RINs of 7.1 ± 0.4 , VAT sample RINs was 5.5 ± 0.4 and neck SAT samples had RINs of 6.8 ± 0.2 .

Gene expression

The gene expression analysis was performed as described in (Rødgaard *et al.* 2012). Briefly, cDNA syntheses of the samples were performed in duplicate (technical replicates) using 500 ng of total RNA. This was reverse transcribed using the QuantiTECT Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. Pre-amplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems).

Primers were designed as described previously in the study by Skovgaard *et al.* (2009). Gene symbols, primer sequences and amplicon lengths are shown in Table S1. Primer amplification efficiencies, dynamic ranges, specificity and sequences were analyzed as described in the study by Rødgaard *et al.* (2012).

Quantitative PCR was performed in 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm), combining 48 samples with 48 primer sets in the BioMark Real-Time PCR instrument (Fluidigm). Expression data were acquired using the Fluidigm REAL-TIME PCR ANALYSIS software 3.0.2 (Fluidigm) and exported to GENEX5 (MultiD, Göteborg, Sweden).

Serum protein quantification

Serum protein concentrations were determined using sandwich enzyme-linked immunosorbent assays (ELISAs). Commercially available ELISA assays were used for interleukin 6 (IL-6) (Porcine IL-6 DuoSet kit, R&D Systems, Inc.), tumor necrosis factor- α (TNF- α) (Swine TNF- α CytoSet™, with Antibody Pair Buffer Kit; Invitrogen) and serum amyloid A (SAA) (Phase SAA assay, Tridelta Development Ltd.) according to manufacturers' protocol. C-reactive protein (CRP) and haptoglobin (HP) were analyzed with in-house assays as described in Rødgaard *et al.* (2012). TMB-plus (Kem-En-Tec) was used for developing the ELISA-

plates, according to the manufacturer's instructions. Plates were then read using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific). ASCENT software v. 2.6 (Thermo Scientific) was used to calculate sample values calibrating each plate with several dilutions of a standard solution of known concentration of the protein in question.

Data analysis and statistics

For qPCR data pre-processing, normalization and relative quantification GENEX5 was used. Out of a panel of five putative reference genes, the most stably expressed were selected for normalization (for liver: *GAPDH*, *B2M*, *RPL13A* and *ACTB*; for the adipose tissues all five were chosen: *HPRT1*, *GAPDH*, *B2M*, *RPL13A* and *ACTB*) using GeNorm (Vandesompele *et al.* 2002). The geometric mean of these was used for normalization of each tissue individually in GENEX5. To calculate relative expression levels, the Cq values were calculated relative to the least expressed sample for each primer set. The data from qPCR and ELISAs were log2 transformed to approach normal distribution prior to two-tailed, unpaired *t*-test, one-way ANOVA with Tukey–Kramer post-test and/or *F*-test, and $P \leq 0.05$ was considered significant. Statistics were performed in GENEX5 or PRISM5 (GraphPad Software, Inc.). Data are expressed as the mean \pm standard error of the mean (SEM).

Results

The mean daily feed intake of the pigs in the obese group having free access to food was around 1.5 kg. At euthanasia, the body weight of the pigs of the obese group (92.6 ± 5.2 kg; mean and SEM respectively) was significantly ($P < 0.0001$) higher than was the body weight in the lean group (50.3 ± 1.6 kg).

Gene expression

The expression profiles of 39 genes related to innate immunity were analyzed as were five putative reference genes. In the pre-processing of data, several genes were removed from further analysis due to low efficiencies or due to high variation between the technical replicates (as described in Rødgaard *et al.* (2012): *FGG*, *IL1B*, *IL8(a)*, *IL8(b)*, *AOAH*, *IL10(b)*, *ORM1(a)*, *ORM1(b)*, *SFTPA1* and *IFNG* were removed from the fatty tissue data and *IL1B*, *AOAH*, *SFTPA1*, *ORM1(a)*, *ORM1(b)*, *ADIPOQ*, *LBP* and *IL8(a)* from the liver tissue data.

Genes differentially expressed between lean and obese pigs at significance level $P = 0.05$ and with a change of at least ± 1.5 -fold are depicted for all four tissues in Fig. 1. Overall, obesity resulted in a significantly changed

expression of 12 genes in VAT (nine of which were down-regulated in obese animals), 11 genes in RPAT (of which seven were up-regulated in the obese group) and eight genes in neck SAT (three of these genes being down-regulated). The *adiponectin* gene was down-regulated in both VAT and RPAT of the obese pigs. The genes significantly correlated with obesity in the liver were mostly down-regulated (six of nine).

Three genes were significantly differentially regulated between the lean and obese minipigs in all three adipose tissues, namely the *chemokine (C-C motif) ligand 3-like 1* (*CCL3L1*), *interleukin 1 receptor antagonist* (*IL1RN*) and *CD200 molecule* (*CD200*), with *CCL3L1* and *IL1RN* being up-regulated and *CD200* being down-regulated in all three adipose tissue types of obese pigs. In the liver, none of these three genes changed expression in obese animals.

The pattern of tissue-specific adipose tissue gene expression also may be compared between lean and obese pigs (see Table 1). For both groups of pigs, approximately half of the investigated genes showed different expression levels in the three types of adipose tissues. In the lean group, all of these genes were expressed at higher levels in RPAT and VAT than in neck SAT (19 genes), whereas in the obese pigs, the number of genes being more highly expressed in RPAT and VAT compared to neck SAT was lower (10 genes). Specifically, in the obese group, more genes encoding for acute phase proteins (APPs) were significantly differentially expressed among adipose tissues, with six genes (*C3*, *CRP*, *HP*, *ITIH4(a)*, *ITIH4(b)*, *LBP* and *SAA*) compared with two genes (*C3* and *CRP*) in the lean minipigs; in contrast, only two genes encoding for cytokines (*TGFB1* and *IL1RN*) and none encoding for chemokines were significantly differentially expressed in adipose tissues in the obese animals, compared with four genes encoding for cytokines (*IL10*, *IL18*, *TGFB1* and *TNFAIP3*) and three encoding chemokines (*CCL3L1* and *CXCL10*) in the adipose tissues of lean minipigs.

Serum protein concentrations

The serum concentrations of HP, CRP and SAA are depicted in Fig. S1. No significant differences in HP, CRP or SAA serum concentrations were found between the lean and obese Göttingen minipigs. The serum levels of TNF- α and IL-6 were below the detection limit (except in one lean minipig in the IL-6 ELISA) and are therefore not depicted. None of the transcripts coding for these proteins were significantly differentially regulated in the liver of obese compared with lean pigs; however, in the VAT, the APPs encoded by *HP* and *SAA* were significantly differentially down-regulated in the obese minipigs compared with the lean minipigs and *IL6* was up-regulated in both the RPAT and the VAT in the obese minipigs compared with the lean (Fig. 1).

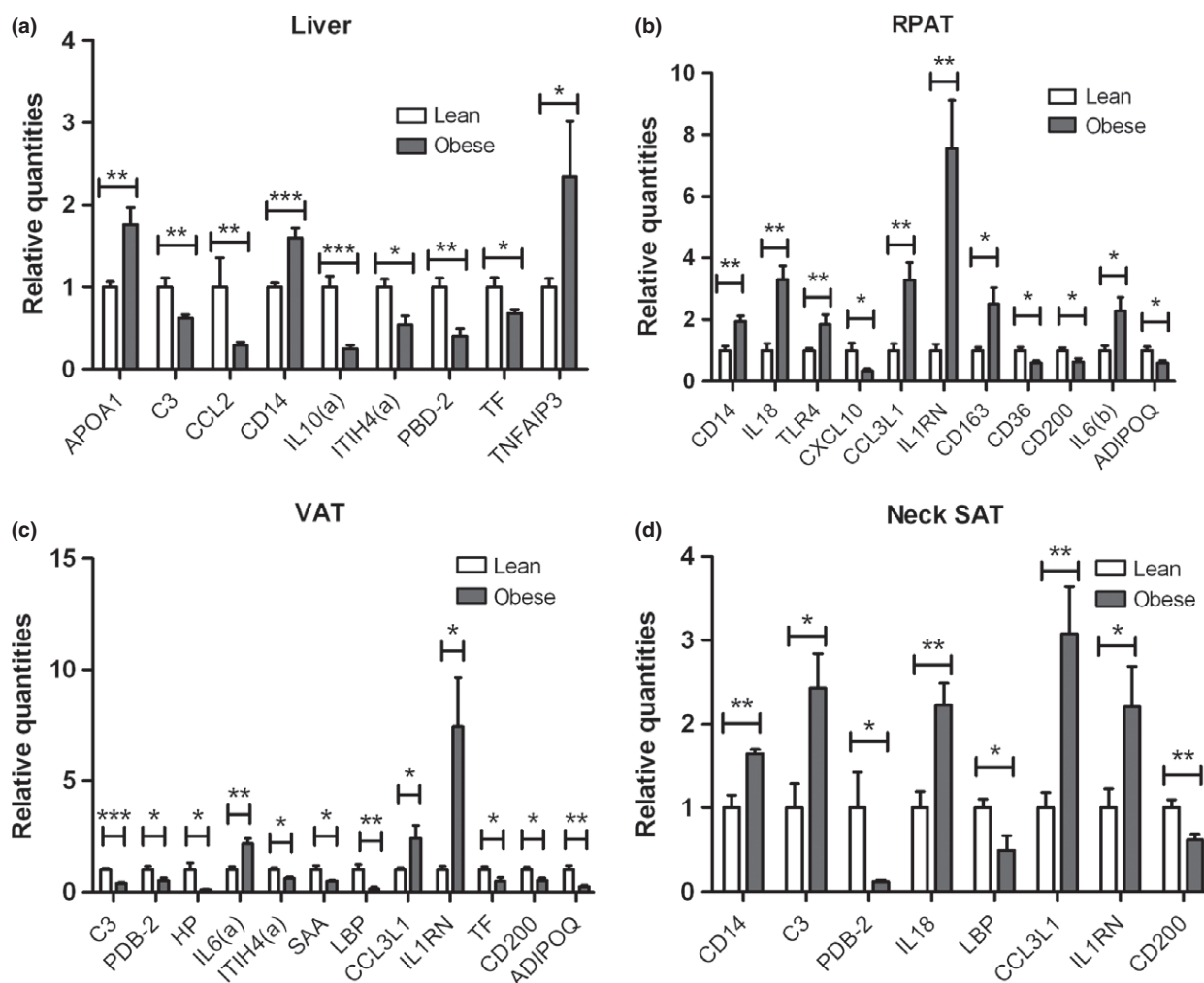


Figure 1 Significantly differentially expressed genes in lean compared with obese Göttingen minipigs. Only genes with a fold change equal to or greater than ± 1.5 [compared with gene expression in lean pigs (set to 1 for each gene)] in liver (a), retroperitoneal adipose tissue (RPAT) (b), visceral adipose tissue (VAT) (c) and neck subcutaneous adipose tissue (SAT) (d) as determined by qPCR are included. In the VAT, both primers for *IL6* showed similar patterns and significance (**); therefore, *IL6(b)* was removed from the graph. For all tissues, lean $n = 7$ and obese $n = 7$. Error bars depict standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Obesity-related changes in the expression of innate immune system-related genes and possible similarities to human obesity of obese Göttingen minipigs have to our knowledge not been thoroughly described before. Here, we studied Göttingen minipigs that were either allowed free access (obese group) or allowed restricted access (lean group) to normal pig chow. Thus, the effects of food intake and obesity cannot be differentiated in this model as they are highly correlated.

The obese Göttingen minipigs had significantly higher bodyweights than did the lean pigs, and we find clear differences in the gene regulation in the obese minipigs compared with lean, including for genes encoding APP and cytokines in adipose tissues (Fig. 1). The expression of a number of other relevant obesity-related genes in

these pigs will be published elsewhere (S. Cirera *et al.* 2013).

Interestingly, three genes were significantly differentially regulated in all three adipose tissues of the obese pigs as compared with the lean pigs, namely *CCL3L1*, *IL1RN* and *CD200* (Fig. 1). *CCL3L1* is up-regulated in the obese minipigs and encodes a chemokine, also known as macrophage inflammatory protein 1- α (MIP-1 α), that is involved in acute inflammation by recruiting and activating polymorphonuclear leukocytes (Wolpe *et al.* 1989). Previous work in mice has established that this gene is up-regulated in the epididymal white adipose tissue of obese mice and may play a role in the development of type II diabetes (Xu *et al.* 2003). To our knowledge, the gene expression of *CCL3L1* has not yet been investigated in human adipose tissues; however, the serum protein concentration is similar in lean and obese humans (Kim *et al.* 2006). The

Table 1 Innate immune gene expression in adipose tissues

Gene	Lean			Obese		
	Tissue			Tissue		
	RPAT	VAT	Neck SAT	RPAT	VAT	Neck SAT
	a	b	c	a	b	c
ADIPOQ ¹	36.75 ± 4.36	19.39 ± 3.97	16.32 ± 2.15	0.11	0.05	1.00
APOA1 ²	2.08 ± 0.32	4.41 ± 0.84	3.91 ± 0.63	0.97	1.00	1.00
C3 ²	10.54 ± 5.14	249.09 ± 20.89	3.71 ± 1.09	<0.001	0.15	<0.001
CCL2 ³	4.19 ± 0.85	5.71 ± 0.93	3.06 ± 0.60	0.87	0.89	0.27
CCL3L1 ³	5.52 ± 1.28	11.36 ± 1.15	6.62 ± 1.23	0.05	0.95	0.28
CCL5 ³	3.67 ± 0.67	5.86 ± 0.42	3.61 ± 0.69	0.19	1.00	0.22
CD14 ⁴	2.22 ± 0.31	4.52 ± 0.71	1.85 ± 0.29	0.005	0.87	<0.001
CD163 ⁴	4.37 ± 0.39	10.02 ± 2.04	1.90 ± 0.34	0.28	0.008	<0.001
CD200 ⁴	6.33 ± 0.54	6.91 ± 0.85	2.52 ± 0.25	1.00	0.03	0.02
CD36 ⁴	5.73 ± 0.56	2.16 ± 0.38	2.64 ± 0.23	<0.001	<0.001	0.74
CD40 ⁴	2.35 ± 0.27	4.68 ± 0.21	1.44 ± 0.15	0.002	0.03	<0.001
COX-2 ⁴	4.54 ± 0.57	6.65 ± 0.52	2.97 ± 0.47	0.63	0.50	0.02
CRP ²	11.38 ± 2.64	7.11 ± 1.26	3.44 ± 0.59	0.86	0.03	0.34
CXC10 ³	18.41 ± 4.55	17.90 ± 4.26	2.91 ± 0.55	1.00	0.01	0.01
HP ²	241.31 ± 215.68	59.36 ± 19.47	10.18 ± 2.56	1.00	0.47	0.48
IL10(a) ⁵	9.10 ± 1.33	18.15 ± 3.56	3.40 ± 0.54	0.92	0.05	0.003
IL18 ⁵	5.35 ± 1.18	9.78 ± 2.30	3.00 ± 0.59	0.98	0.19	0.04
IL1RN ⁵	23.60 ± 5.07	68.60 ± 11.30	30.92 ± 7.18	0.15	1.00	0.16
IL6(a) ⁵	3.37 ± 0.64	2.36 ± 0.35	2.49 ± 0.23	0.84	0.95	1.00
IL6(b) ⁵	3.66 ± 0.56	2.38 ± 0.37	2.50 ± 0.35	0.70	0.81	1.00
ITIH4(a) ²	3.98 ± 0.89	2.45 ± 0.27	2.63 ± 0.37	0.70	0.51	1.00
ITIH4(b) ²	3.85 ± 1.03	3.31 ± 0.25	4.35 ± 0.55	0.96	1.00	0.99
LRP ²	197.03 ± 85.54	65.79 ± 16.09	85.56 ± 9.09	0.55	0.74	1.00
NFKB1 ⁴	2.19 ± 0.13	2.98 ± 0.22	1.54 ± 0.11	0.46	0.004	<0.001
NFKBIA ⁴	2.31 ± 0.21	2.69 ± 0.24	1.45 ± 0.16	1.00	0.03	0.008
PAFAH1B1 ⁴	1.71 ± 0.08	1.64 ± 0.06	1.32 ± 0.08	0.85	0.004	0.08
PDB-2 ⁴	311.91 ± 107.09	351.50 ± 58.67	906.21 ± 381.26	1.00	0.83	0.86
SAA ²	14.01 ± 5.49	9.21 ± 1.89	4.60 ± 1.37	1.00	0.49	0.30
TF ²	62.37 ± 56.27	63.68 ± 9.22	12.55 ± 3.48	0.85	0.99	0.46
TGFB1 ⁵	2.87 ± 0.20	5.86 ± 0.54	1.77 ± 0.20	<0.001	0.001	<0.001
TLR4 ⁴	1.63 ± 0.11	3.74 ± 0.22	2.14 ± 0.10	0.02	1.00	0.02
TNF ⁵	61.12 ± 13.59	118.16 ± 24.56	35.74 ± 5.73	0.18	1.00	0.10
TNFAIP3 ⁵	2.02 ± 0.31	2.67 ± 0.15	1.53 ± 0.14	0.49	0.81	0.05

(a) Significance of difference in expression of gene in RPAT compared with expression in VAT. (b) Significance of difference in expression of gene in RPAT compared with expression in neck SAT. (c) Significance of difference in expression of gene in VAT compared with expression in neck SAT.

¹Adipokines.²Acute phase proteins.³Chemokines.⁴Immune regulatory genes.⁵Cytokines.

up-regulation of this pro-inflammatory gene (*CCL3L1*) is accompanied by the down-regulation of the anti-inflammatory gene *CD200*. *CD200* is a receptor on the surface of macrophages, for example, and helps down-regulate myeloid cell activity (Hoek *et al.* 2000; Wright *et al.* 2000). The combined effect of these changes in the adipose tissues of the obese minipigs compared with the lean would be pro-inflammatory. However, the most up-regulated gene in VAT and RPAT of obese pigs is *IL1RN*. The product of *IL1RN* is the interleukin 1 receptor antagonist (IL1ra), a naturally occurring inhibitor of the pro-inflammatory proteins IL-1 α and - β (Seckinger *et al.* 1987). White adipose tissue has been found to be a major source of IL1ra in both mice and men (Juge-Aubry *et al.* 2003), and IL1ra may play a role in acquired resistance to leptin in obesity in rats and humans (Luheshi *et al.* 1999; Meier *et al.* 2002). *IL1RN* is significantly up-regulated in the VAT compared with neck SAT in the obese pigs ($P = 0.002$) (Table 1), which could counteract the pro-inflammatory effects of the down-regulated *CD200* and the up-regulated *CCL3L1*. This might explain the absence of changes in serum cytokine and APP concentrations in the obese Göttingen minipigs studied here. Interestingly, *IL6* is up-regulated in the RPAT and VAT in the obese minipigs, and previous reports suggest that IL6 suppresses inflammation via induction of IL1ra synthesis in humans (Tilg *et al.* 1994). This is supported by the findings that the up-regulation of *IL1RN* expression in the RPAT and VAT, where *IL6* is up-regulated, is much higher than in the neck SAT (7.5 times higher in the RPAT and VAT of the obese pigs and only two times higher in the neck SAT of the obese minipigs compared to lean) (Fig. 1b–d).

A correlation between increased adipose tissue expression and elevated serum levels of various innate immune factors in obesity has been established in humans for a number of proteins, including IL-6 (Fontana *et al.* 2007), HP (Chiellini *et al.* 2004) and SAA (Poitou *et al.* 2006). However, such correlations were not found in the Göttingen minipigs investigated in the present study. Of the three genes mentioned, adipose tissue expression of only *IL6* was elevated, in both the RPAT and the VAT (Fig. 1); however, no increases in serum concentration of IL-6 were observed in the Göttingen minipigs. The expression of both SAA and HP was down-regulated in the VAT, and no changes in serum concentrations of these proteins were detected either. This contrasts with the serum protein changes observed in the human response to obesity, in which CRP, SAA and HP serum concentrations are elevated (van Dielen *et al.* 2001; Esposito *et al.* 2003; Chiellini *et al.* 2004). A reason for this could be that the fold changes of the genes significantly correlated with obesity in the liver are very small (Fig. 1a) and perhaps too small for the changes to be translated into elevated serum protein concentration. Another reason why the Göttingen minipigs in this study do not show a serum response to obesity similar to humans could be that their

diet contained no excess fat or sugar, and it is possible that a standard diet does not induce a systemic or local low-grade inflammation. However, the pigs had been obese for a prolonged period of time, as they were euthanized at 41–47 months, which mimics the human chronic obesity state.

In summary, a wide range of innate immunology-related genes are significantly differentially regulated in the obese Göttingen minipigs compared with the lean pigs. However, in contrast to human obesity, this does not translate into changes in serum concentrations of cytokines and APPs in the obese pigs compared with the lean. This should be taken into account when selecting biomarkers for obesity in Göttingen minipigs.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Serum protein concentrations.

Table S1 Primers used for high-throughput qPCR.